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2

Molecular Cloning

A LABORATORY MANUAL

SECOND EDITION

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BRIDIZATION OF RADIOLABELED PROBES IMMOBILIZED NUCLEIC ACIDS

There are many methods available to hybridize radioactive probes in solution to nucleic acids immobilized on solid supports such as nitrocellulose filters or nylon membranes. These methods differ in the following respects:

- Solvent and temperature used (e.g., 68°C in aqueous solution or 42°C in 50% formamide)
- Volume of solvent and length of hybridization (large volumes for periods as long as 3 days or minimal volumes for periods as short as 4 hours)
- Degree and method of agitation (continuous shaking or stationary)
- Use of agents such as Denhardt's reagent or BLOTTO to block the non-specific attachment of the probe to the surface of the solid matrix
- Concentration of the labeled probe and its specific activity
- Use of compounds, such as dextran sulfate (Wahl et al. 1979) or polyethylene glycol (Renz and Kurz 1984; Amasino 1986), that increase the rate of reassociation of nucleic acids
- Stringency of washing following the hybridization

Although the choice depends to a large extent on personal preference, we offer the following guidelines for choosing among the various methods available.

1. Hybridization reactions in 50% formamide at 42°C are less harsh on nitrocellulose filters than is hybridization at 68°C in aqueous solution. However, it has been found that the kinetics of hybridization in 80% formamide are approximately four times slower than in aqueous solution (Casey and Davidson 1977). Assuming a linear relationship between the rate of hybridization and the formamide concentration, the rate in 50% formamide should be two to three times slower than the rate in aqueous solution. Both types of solvents give excellent results and neither has a clear-cut advantage over the other.
2. The smaller the volume of hybridization solution, the better. In small volumes of solution, the kinetics of nucleic acid reassociation are faster and the amount of probe needed can be reduced so that the DNA on the filter acts as the driver for the reaction. However, it is essential that sufficient liquid be present for the filters to remain covered at all times by a film of the hybridization solution.
3. Continual movement of the probe solution across the filter is unnecessary, even for a reaction driven by the DNA immobilized on the filter. However, if a large number of filters are hybridized simultaneously, agitation is advisable to prevent the filters from adhering to one another.
4. The kinetics of the hybridization reaction are difficult to predict from theoretical considerations, partly because the exact concentration of the

immobilized nucleic acid and its availability for hybridization are unknown. When using probes that have the capacity to self-anneal (e.g., nick-translated double-stranded DNA), the following rule of thumb is useful: Allow the hybridization to proceed for a time sufficient to enable the probe in solution to achieve $1–3 \times C_o t_{1/2}$. In 10 ml of hybridization solution, 1 μg of a probe of 5-kb complexity will reach $C_o t_{1/2}$ in 2 hours. To determine the time of half-renaturation for any other probe, simply enter the appropriate values into the following equation:

$$1/x \times y/5 \times z/10 \times 2 = \text{number of hours to achieve } C_o t_{1/2}$$

where x = the weight of the probe added (in micrograms), y = its complexity (for most probes, complexity is proportional to the length of the probe in kilobases), and z = the volume of the reaction (in milliliters).

After hybridization to $3 \times C_o t_{1/2}$ has been reached, the amount of probe available for additional hybridization to the filter is negligible. For probes that do not have the capacity to self-anneal (e.g., RNA probes synthesized in vitro by bacteriophage-encoded DNA-dependent RNA polymerases; see Chapter 10), the hybridization time may be shortened, since the lack of a competing reaction in the solution favors hybridization of the probe to the DNA immobilized on the filter.

5. Several different types of agents can be used to block the nonspecific attachment of the probe to the surface of the filter. These include Denhardt's reagent (Denhardt 1966), heparin (Singh and Jones 1984), and nonfat dried milk (Johnson et al. 1984). Frequently, these agents are used in combination with denatured, fragmented salmon sperm or yeast DNA and detergents such as SDS. In our experience, virtually complete suppression of background hybridization is obtained by prehybridizing filters with a blocking agent consisting of 5 × Denhardt's reagent, 0.5% SDS, and 100 μg/ml denatured, fragmented DNA. We recommend this mixture whenever the signal-to-noise ratio is expected to be low, for example, when carrying out northern analysis of low-abundance mRNAs or Southern hybridizations with single-copy sequences of mammalian DNA. However, in most other circumstances (Grunstein/Hogness hybridization [1975], Benton/Davis hybridization [1977], Southern hybridization [1975] of abundant DNA sequences, etc.), we recommend using 0.25% nonfat dried milk (0.05 × BLOTTO; Johnson et al. 1984). This is much less expensive, easier to use than Denhardt's reagent, and, for these purposes, gives results that are equally satisfactory. In general, Denhardt's reagent is more effective for nylon membranes. The signal-to-noise ratio obtained with most brands of nylon membranes is higher with Denhardt's reagent than with BLOTTO. Nonfat dried milk is not recommended when using RNA probes or when carrying out northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase activity. For more information about blocking agents, see Table 9.1.

6. Blocking agents are usually included in both the prehybridization and hybridization solutions when nitrocellulose filters are used. However, when the nucleic acid is immobilized on nylon membranes, the blocking agents are often omitted from the hybridization solution, since high

TABLE 9.1 Blocking Agents Used to Suppress Background in Hybridization Experiments

Agent	Recommended uses
Denhardt's reagent	northern hybridizations hybridizations using RNA probes single-copy Southern hybridizations hybridizations involving DNA immobilized on nylon membranes

Denhardt's reagent (Denhardt 1966) is usually made up as a 50× stock solution, which is filtered and stored at −20°C. The stock solution is diluted tenfold into prehybridization buffer (usually 6× SSC or 6× SSPE containing 0.5% SDS and 100 μg/ml denatured, fragmented, salmon sperm DNA). 50× Denhardt's reagent contains 5 g of Ficoll (Type 400, Pharmacia) 5 g of polyvinylpyrrolidone, 5 g of bovine serum albumin (Pentex Fraction V), and H_2O to 500 ml.

BLOTTO	Grunstein/Hogness hybridization Benton/Davis hybridization all Southern hybridizations other than single-copy dot blots

1× BLOTTO (Bovine Lacto Transfer Technique Optimizer; Johnson et al. 1984) is 5% non-fat dried milk dissolved in water containing 0.02% sodium azide. It should be stored at 4°C and diluted 25-fold into hybridization buffer before use. BLOTTO should not be used in combination with high concentrations of SDS, which will cause the milk proteins to precipitate. If background hybridization is a problem, NP-40 may be added to the hybridization solution to a final concentration of 1%. BLOTTO should not be used as a blocking agent in northern hybridizations because of the possibility that it might contain unacceptably high levels of RNAase.

Caution: Sodium azide is poisonous. It should be handled with great care, wearing gloves, and solutions containing it should be clearly marked.

Heparin	Southern hybridization in situ hybridization

Heparin (Sigma H-7005 porcine grade II or equivalent) is dissolved at a concentration of 50 mg/ml in 4× SSPE or 4× SSC and stored at 4°C. It is used as a blocking agent at a concentration of 500 μg/ml in hybridization solutions containing dextran sulfate; in hybridization solutions that do not contain dextran sulfate, heparin is used at a concentration of 50 μg/ml (Singh and Jones, 1984).

Denatured, fragmented salmon sperm DNA	Southern and northern hybridizations

Salmon sperm DNA (Sigma type III sodium salt) is dissolved in water at a concentration of 10 mg/ml. If necessary, the solution is stirred on a magnetic stirrer for 2–4 hours at room temperature to help the DNA to dissolve. The solution is adjusted to 0.1 M NaCl and extracted once with phenol and once with phenol:chloroform. The aqueous phase is recovered and the DNA is sheared by passing it 12 times rapidly through a 17-gauge hypodermic needle. The DNA is precipitated by adding 2 volumes of ice-cold ethanol. It is then recovered by centrifugation and redissolved at a concentration of 10 mg/ml in water. The OD^{260} of the solution is determined and the exact concentration of the DNA is calculated. The solution is then boiled for 10 minutes and stored at −20°C in small aliquots. Just before use, the solution is heated for 5 minutes in a boiling-water bath and then chilled quickly in ice water. Denatured, fragmented salmon sperm DNA should be used at a concentration of 100 μg/ml in hybridization solutions.

concentrations of protein are believed to interfere with the annealing of the probe to its target. This quenching of the hybridization signal is particularly noticeable when oligonucleotides or probes less than 100 nucleotides in length are used.

7. In the presence of 10% dextran sulfate or 10% polyethylene glycol, the rate of hybridization is accelerated approximately tenfold (Wahl et al. 1979; Renz and Kurz 1984; Amasino 1986) because nucleic acids are excluded from the volume of the solution occupied by the polymer and their effective concentration is therefore increased. Although dextran sulfate and polyethylene glycol are useful in circumstances where the rate of hybridization is the limiting factor in detecting rare sequences (e.g., northern or genomic Southern blots), they are of no benefit when screening bacterial colonies or bacterial plaques. In addition, they can sometimes lead to high backgrounds, and hybridization solutions containing them are always difficult to handle because of their viscosity. We therefore recommend that dextran sulfate and polyethylene glycol not be used unless the rate of hybridization is very slow, the filter contains very small amounts of DNA, or the amount of radiolabeled probe is limiting.

8. To maximize the rate of annealing of the probe with its target, hybridizations are usually carried out in solutions of high ionic strength (6 × SSC or 6 × SSPE) at a temperature that is 20–25°C below the melting temperature (T_m). Both solutions work equally well when hybridization is carried out in aqueous solvents. However, when formamide is included in the hybridization buffer, 6 × SSPE is preferred because of its greater buffering power.

9. In general, the washing conditions should be as stringent as possible (i.e., a combination of temperature and salt concentration should be chosen that is approximately 12–20°C below the calculated T_m of the hybrid under study). The temperature and salt conditions can often be determined empirically in preliminary experiments in which samples of genomic DNA immobilized on filters are hybridized to the probe of interest and then washed under conditions of different stringencies.

10. To minimize background problems, it is best to hybridize for the shortest possible time using the minimum amount of probe. For Southern hybridization of mammalian genomic DNA where each lane of the gel contains 10 μg of DNA, 10–20 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater) should be used and hybridization should be carried out for 12–16 hours at 68°C in aqueous solution or for 24 hours at 42°C in 50% formamide. For Southern hybridization of fragments of cloned DNA where each band of the restriction digest contains 10 ng of DNA or more, much less probe is required. Typically, hybridization is carried out for 6–8 hours using 1–2 ng/ml radiolabeled probe (sp. act. = 10^9 cpm/μg or greater).

11. *Useful facts*:

    a. The T_m of the hybrid formed between the probe and its target may be estimated from the following equation (Bolton and McCarthy 1962):

$$T_m = 81.5°C - 16.6(\log_{10}[Na^+]) + 0.41(\%\,G + C) - 0.63(\%\,\text{formamide}) - (600/l)$$

where l = the length of the hybrid in base pairs.

This equation is valid for:

- Concentrations of Na^+ in the range of 0.01 M to 0.4 M. It predicts T_m less accurately in solutions of higher $[Na^+]$.
- DNAs whose G + C content is in the range of 30% to 75%. Note that the depression of T_m in solutions containing formamide is greater for poly(dA:dT) (0.75°C/1% formamide) and less for DNAs rich in poly(dG:dC) (0.50°C/1% formamide) (Casey and Davidson 1977).

The equation applies to the "reversible" T_m that is defined by optical measurement of hyperchromicity at OD_{257}. The "irreversible" T_m, which is more important for autoradiographic detection of DNA hybrids, is usually 7–10°C higher than that predicted by the equation.

Similar equations have been derived for:

i. RNA probes hybridizing to immobilized RNA (Bodkin and Knudson 1985)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\%G + C) + 11.8(\%G + C)^2 - 0.35(\%\,\text{formamide}) - (820/l)$$

ii. DNA:RNA hybrids (Casey and Davidson 1977)

$$T_m = 79.8°C + 18.5(\log_{10}[Na^+]) + 0.58(\%G + C) + 11.8(\%G + C)^2 - 0.50(\%\,\text{formamide}) - (820/l)$$

Comparison of these equations shows that the relative stability of nucleic acid hybrids decreases in the following order: RNA:RNA (most stable), RNA:DNA (less stable), and DNA:DNA (least stable). In aqueous solutions, the T_m of a DNA:DNA hybrid is approximately 10°C lower than that of the equivalent RNA:RNA hybrid. In 80% formamide, the T_m of an RNA:DNA hybrid is approximately 10°C higher than that of the equivalent DNA:DNA hybrid.

b. The T_m of a double-stranded DNA decreases by 1–1.5°C with every 1% decrease in homology (Bonner et al. 1973).

The above equations apply only to hybrids greater than 100 nucleotides in length. The behavior of oligonucleotide probes is described in detail in Chapter 11.

For a general discussion of hybridization of nucleic acids bound to solid supports, see Meinkoth and Wahl (1984).

BIOCHEMISTRY

THIRD EDITION

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The distribution of ^{14}N and ^{15}N was revealed by the newly developed technique of *density-gradient equilibrium sedimentation.* A small amount of DNA was dissolved in a concentrated solution of cesium chloride having a density close to that of the DNA ($\sim$1.7 g cm^{-3}). This solution was centrifuged until it was nearly at equilibrium. The opposing processes of sedimentation and diffusion created a gradient in the concentration of cesium chloride across the centrifuge cell. The result was a stable density gradient, ranging from 1.66 to 1.76 g cm^{-3}. The DNA molecules in this density gradient were driven by centrifugal force into the region where the solution's density was equal to their own. High-molecular-weight DNA yielded a narrow band that was detected by its absorption of ultraviolet light. A mixture of ^{14}N DNA and ^{15}N DNA molecules gave clearly separate bands because they differ in density by about 1% (Figure 4-13).

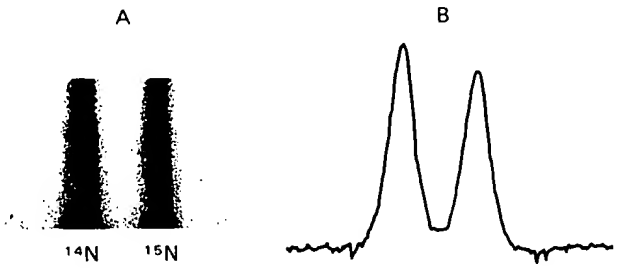

Figure 4-13
Resolution of ^{14}N DNA and ^{15}N DNA by density-gradient centrifugation: (A) ultraviolet absorption photograph of a centrifuge cell; (B) densitometric tracing of the absorption photograph. [From M. Meselson and F. W. Stahl. *Proc. Nat. Acad. Sci.* 44(1958):671.]

DNA was extracted from the bacteria at various times after they were transferred from a ^{15}N to a ^{14}N medium. Analysis of these samples by the density-gradient technique showed that there was a single band of DNA after one generation (Figure 4-14). The density of this band was precisely halfway between those of ^{14}N DNA and ^{15}N DNA. *The absence of ^{15}N DNA indicated that parental DNA was not preserved as an intact unit on replication.* The absence of ^{14}N DNA indicated that all of the daughter DNA molecules derived some of their atoms from the parent DNA. This proportion had to be one-half, because the density of the hybrid DNA band was halfway between those of ^{14}N DNA and ^{15}N DNA.

After two generations, there were equal amounts of two bands of DNA. One was hybrid DNA, the other was ^{14}N DNA. Meselson and Stahl concluded from these incisive experiments *"that the nitrogen of a DNA molecule is divided equally between two physically continuous subunits that, following duplication, each daughter molecule receives one of these; and that the subunits are conserved through many duplications."* Their result agreed perfectly with the Watson-Crick model for DNA replication (Figure 4-15).

THE DOUBLE HELIX CAN BE REVERSIBLY MELTED

The two strands of a DNA helix readily come apart when the hydrogen bonds between its paired bases are disrupted. This can be accomplished

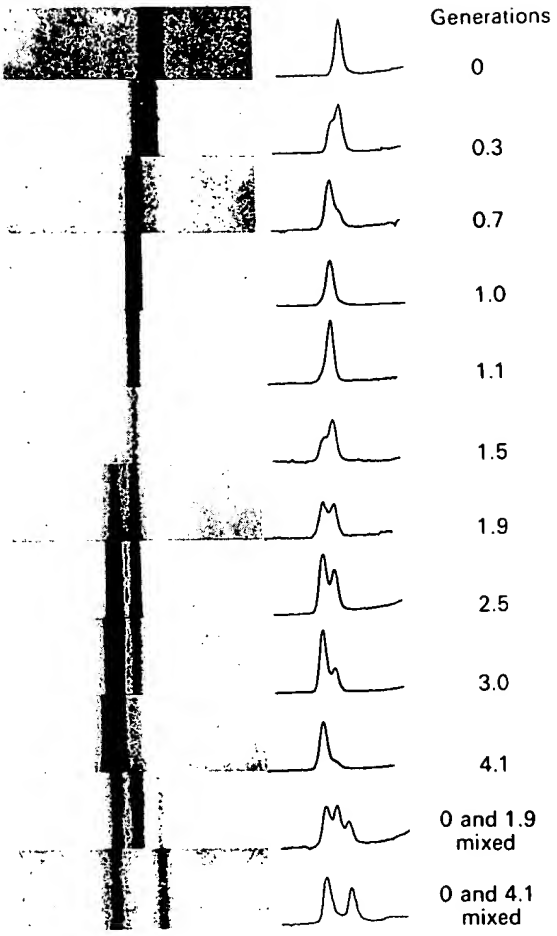

Figure 4-14
Detection of semiconservative replication in *E. coli* by density-gradient centrifugation. The position of a band of DNA depends on its content of ^{14}N and ^{15}N. After 1.0 generation, all of the DNA molecules are hybrids containing equal amounts of ^{14}N and ^{15}N. No parental DNA (^{15}N) is left after 1.0 generation. [From M. Meselson and F. W. Stahl. *Proc. Nat. Acad. Sci.* 44(1958):671.]

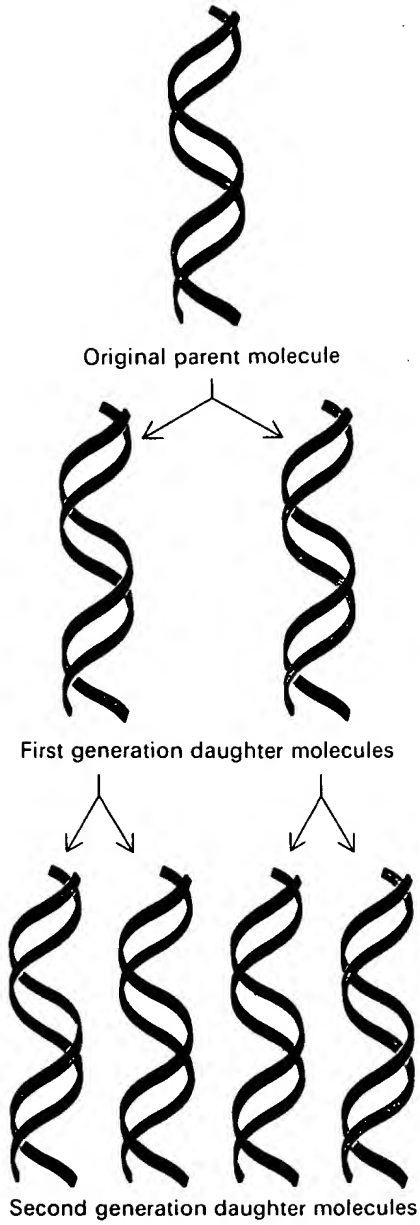

Figure 4-15
Schematic diagram of semiconservative replication. Parental DNA is shown in green and newly synthesized DNA in red. [After M. Meselson and F. W. Stahl. *Proc. Nat. Acad. Sci.* 44(1958):671.]

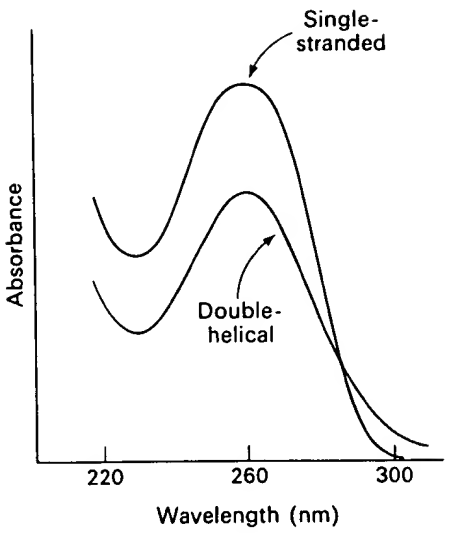

Figure 4-16
The absorbance of a DNA solution at wavelength 260 nm increases when the double helix is melted into single strands.

by heating a solution of DNA or by adding acid or alkali to ionize it bases. The unwinding of the double helix is called *melting* because i occurs abruptly at a certain temperature. The *melting temperature* (T_m) i defined as the temperature at which half of the helical structure is lost The abruptness of the transition indicates that the DNA double helix i a *highly cooperative structure*, held together by many reinforcing bonds; i is stabilized by the stacking of bases as well as by base pairing. Th melting of DNA is readily monitored by measuring its absorbance o light at wavelength 260 nm. The unstacking of the base pairs results i increased absorbance, an effect called *hyperchromism* (Figure 4-16).

The melting temperature of a DNA molecule depends markedly o its base composition. DNA molecules rich in GC base pairs have higher T_m than those having an abundance of AT base pairs (Figur 4-17). In fact, the T_m of DNA from many species varies linearly with G

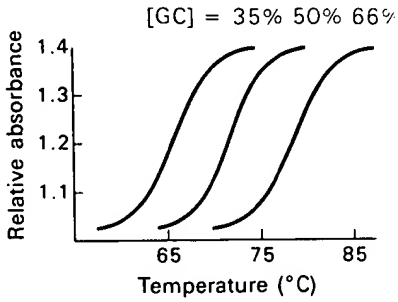

Figure 4-17
DNA melting curves. The absorbance relative to that at 25°C is plotted against temperature. (The wavelength of the incident light was 260 nm.) The T_m is 69°C for *E. coli* DNA (50% GC pairs) and 76°C for *P. aeruginosa* DNA (68% GC pairs).

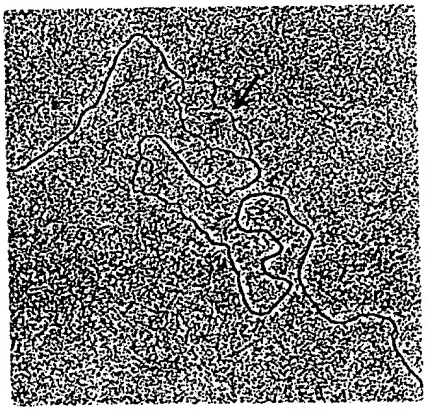

Figure 4-18
Electron micrograph of a DNA molecule partly unwound by alkali. Single-stranded regions appear as loops that stain less intensely than double-stranded segments. These unwound regions are rich in AT base pairs. One of them is marked by an arrow. [From R. B. Inman and M. Schnos. *J. Mol. Biol.* 49(1970):93.]

content, rising from 77°C to 100°C as the fraction of GC pairs increas from 20% to 78%. GC base pairs are more stable than AT pairs becau their bases are held together by three hydrogen bonds rather than two. In addition, adjacent GC base pairs interact more strongly w one another than do adjacent AT base pairs. Hence, *the AT-rich regi of DNA are the first to melt* (Figure 4-18). The double helix is melted vivo by the action of specific proteins (p. 672).

Separated complementary strands of DNA spontaneously reassoci to form a double helix when the temperature is lowered below T_m. T renaturation process is sometimes called *annealing*. The facility w which double helices can be melted and then reassociated is crucial the biological functions of DNA.

DNA MOLECULES ARE VERY LONG

A striking characteristic of naturally occurring DNA molecules is tl length. DNA molecules must be very long to encode the large num of proteins present in even the simplest cells. The *E. coli* chromoso for example, is a single molecule of double-helical DNA consisting four million base pairs. The mass of this DNA molecule is 2.6×10^6 It has a *highly asymmetric shape* when taken out of the cell. The lengtl *E. coli* DNA is 14×10^6 Å, but its diameter is only 20 Å. The 1.4-1 length of this DNA molecule corresponds to a macroscopic dimens whereas its width of 20 Å is on the atomic scale. Bruno Zimm fo that the largest chromosome of *Drosophila melanogaster* contains a si DNA molecule of 6.2×10^7 base pairs, which has a length of 2.1